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(54) Protein expression system.

(57) An expression system for producing and isolating large quantities of protein. This system employs an expression vector, comprising (a) a coding region for a glutathione-binding polypeptide (glutathione-s-transferase preferred), operatively connected to a promoter, (b) a second coding region in-frame with the first coding region, and (c) at least one restriction site between the first and second coding regions wherein a fusion protein of the first and second coding regions will result from expression of the vector. This vector is used in a host cell, which in turn is used in a process for isolating and purifying a protein. This process comprises (a) treating the host cell under conditions allowing expression of the vector, whereby a fusion protein of the first and second coding regions will be expressed; (b) exposing proteins from the host cell to glutathione resin, whereby the fusion protein will adhere to the resin; and (c) cleaving the expression product of the second coding region from the resin. Also described is a process for expressing a nucleic acid sequence, which comprises (a) inserting the nucleic acid sequence into a baculovirus expression vector in-frame with the first coding region; (b) placing the vector into a host cell; (c) treating the host cell under conditions allowing expression of the vector, resulting in expression of a fusion protein of the first coding region and the sequence inserted in step (a); (d) exposing proteins from the host cell to glutathione resin, whereby the fusion protein will adhere to the resin; and (e) treating the adhered fusion protein with a protease to release the expression product of the nucleic acid sequence from the resin. A baculovirus/Spodoptera frugiperda expression system is preferred.

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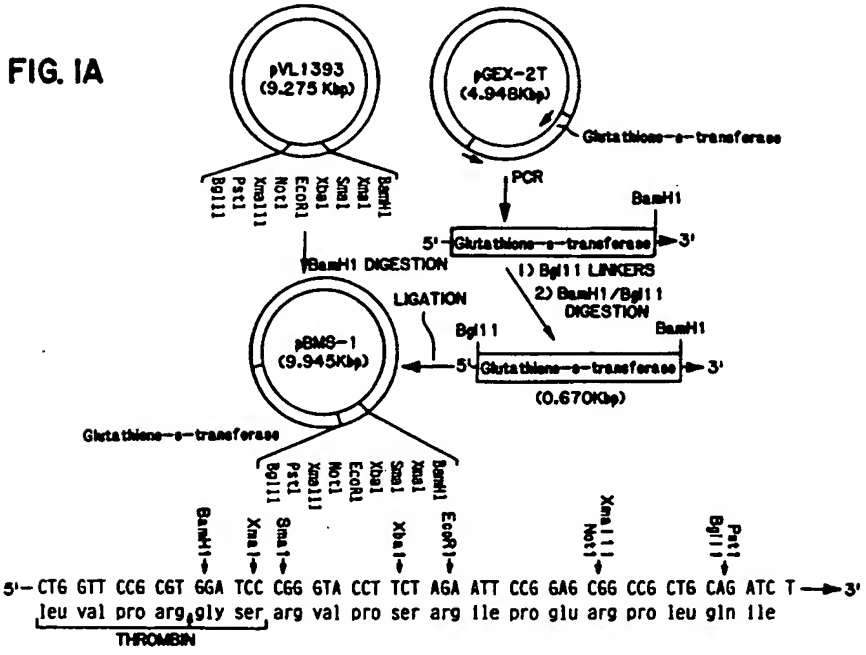
FIG. IA


pVL1393
(9.275 Kbp)
pGEX-2T
(4.948Kbp)
Glutathione-s-transferase
PCR
BamH1
5'
Glutathione-s-transferase
3'
BamH1 DIGESTION
1) Bgl11 LINKERS
2) BamH1/Bgl11
DIGESTION
LIGATION
pBMS-1
(9.945Kbp)
Bgl11
BamH1
(0.670Kbp)
BamH1
Xma1
Sma1
Xba1
EcoR1
Xma111
Not1
Pst1
Bgl11
5'-CTG GTT CCG CGT GGA TCC CGG GTA CCT TCT AGA ATT CCG GAG CGG CCG CTG CAG ATC T-3'
leu val pro arg gly ser arg val pro ser arg ile pro glu arg pro leu gln ile
THROMBIN

The present invention relates to processes for expression of proteins and to xpr ssion vectors and host cells used ther for.

The lck gene product, $p56^{lck}$, is a member of the src family of protein tyrosine kinases. Cooper, J.A. (1990) in Peptides and Protein Phosphorylation (Kemps, B.E., ed) pp. 85-113, CRC Press, Boca Raton, FL.. The lck protein is normally expressed in T lymphocytes and natural killer cells, where it likely performs a variety of functions relating to signal transduction through ligand binding to selected surface proteins. Bolen, J.A., and Veillette, A. (1989) Trends Biochem. Sci. **14**, 404- 407; Rudd, C.E. (1990) Immunol. Today **11**, 400-406. In T-cells, $p56^{lck}$ forms a non-covalent complex with the CD4 and CD8a. Veillette, A., Bookman, M.A., Horak, E.M., and Bolen, J.A. (1988). For this reason, $p56^{lck}$ is believed to aid in mediation of signals emanating from the T-cell antigen receptor through ligation of CD4 or CD8 to non-polymorphic determinants on antigen-bearing major histocompatibility molecules. Shaw, A.S., Chalupny, J., Whitney, J.A., Hammond, C., Amrein, K.E., Kavathas, P., Sefton, B.M., and Rose, J.K., (1990) Mol. Cell. Biol. **10**, 1853-1862; Doyle, C., and Strominger, J.L. (1987) Nature **330**, 256-259; Norment, A.M., Salter, R.D., Parham, P., Engelhard, V.H., and Littman, D.R. (1988) Nature **336**, 79-81. More recently, $p56^{lck}$ has been implicated as a signaling component of the high affinity interleukin-2 receptor. Hatakeyama, M., Kono, T., Kobayashi, N., Kawahara, A., Levin, S.D., Perlmutter, R.M., and Tanaguchi, T. (1991) Science **252**, 1523-1528.

A better understanding of the structure and regulation of $p56^{lck}$ and similar proteins would clearly contribute to our knowledge of early signal transduction events and a source of large quantities of purified $p56^{lck}$ would be useful. While early analysis of $p56^{lck}$ functions have been greatly facilitated by antibodies directed against this protein, immunoaffinity purification has been hampered by lack of an abundant source of enzyme. This difficulty has been addressed in part by baculovirus expression systems. Summers, M.D., and Smith, G.E. (1987). A Manual for baculovirus vectors and insect cell culture procedures, Texas A&M bulletin No. 1555, (College Station, Texas Agricultural Experimental Station and Texas A&M University), 10-39. Recent studies using a baculovirus expression system have reported significant purification of $p56^{lck}$ using conventional chromotography methodologies. Ramer S.E., Winkler, D.G., Carrera, A., Roberts, T.M., and Walsh, C.T. (1991) Proc. Natl. Acad. Sci. USA **88**, 6254-6258; Watts, J.D., Wilson, G.M., Ettehadieh, E., Clark-Lewis, I., Kubanek, C., Astell, C.R., Marth, J.D., and Aebersold, R, (1991) J. Biol. Chem. **267**, 901-907. While this approach results in purified enzyme, multiple column enzyme purification is costly, time-consuming, and requires large amounts of starting material.

Glutathione-s-transferase (Gst) is a protein well known to bind to glutathione (Smith, D.B., and Johnson, K.S. (1988) Gene **67**, 31-40). Glutathione resin may be used in column chromatography. The above baculovirus expression systems, however, do not employ Gst.

The present invention relates to processes for expressing isolated forms of proteins and to expression vectors and host cells useful for such processes. In particular, this invention relates to an expression vector, comprising:

(a) a first coding region, which codes for a polypeptide capable of binding to gluthathione, operatively connected to a promoter,

(b) a second coding region in-frame with the first coding region, and

(c) at least one restriction site between the first and second coding regions;

wherein a fusion protein of the first and second coding regions would result from expression of the vector. Vectors derived from baculovirus are preferred.

Further in accordance with this invention is a host cell comprising such a vector. The preferred host cell is a Spodoptera frugiperda cell, particularly an Sf9 cell, although other host cells are suitable (see below).

Such vectors and host cells are useful in a process for expressing a protein in isolated form, which comprises:

(a) treating such a host cell under conditions allowing expression of the vector, whereby a fusion protein of the first and second coding regions will be expressed;

(b) exposing proteins from the host cell to glutathione resin, whereby the fusion protein will adhere to the resin; and

(c) clearing the expression product of the second coding region from the resin-bound fusion protein.

Further in accordance with the present invention is a process for expressing a nucleic acid sequence, which comprises:

(a) inserting the nucleic acid sequence into a baculovirus expression vector in-frame with a first coding region for a polypeptid capable of binding to glutathione, wherein the coding region is operatively linked to a promoter;

(b) placing the vector into a host cell;

(c) treating the host cell under conditions allowing xpr ssion of the vector, resulting in expression of a fusion protein of the first coding region and th sequ nce inserted in step (a);

(d) exposing proteins from the host cell to glutathione resin, whereby the fusion protein adh res to the resin; and

(e) treating the adhered fusion protein with a protease to release the expression product of the nucleic acid sequence from the resin.

For the first coding region, the inventors prefer a sequence encoding glutathione-s-transferase (nucleotide SEQ. ID. NO.: 1; amino acid SEQ. ID. NO.: 2) or a fragment thereof capable of binding to glutathione. This system combines the high level expression of foreign proteins with baculovirus vectors (e.g., in Sf9 cells) and the ability of Gst fusion proteins to bind to glutathione resin. Treatment of the glutathione-binding fusion protein with a proteolytic substance such as thrombin can thus liberate the desired protein from the glutathione-binding portion of the fusion protein. The glutathione-binding portion remains bound to the resin, thus purifying the desired protein.

This expression system presents advantages over other systems, because it allows the practitioner (1) to produce large quantities of protein, (2) to purify significant amounts of active protein by a single chromatography step, (3) to use a wide range of extraction conditions, including non-denaturing detergents to maintain protein function, (4) to use anti-Gst antibodies, allowing for screening of recombinant baculoviruses that express cloned sequences to which antibodies have not been generated or proteins whose function can not be measured, (5) to use a multiple cloning site with many restriction sites for convenient ligation, and (6) to use and/or study thrombin because it includes a thrombin cleavage site.

The following definitions apply to the terms as used throughout this specification, unless otherwise limited in specific instances.

The term "fusion protein" refers to a protein or polypeptide that has an amino acid sequence having portions corresponding to amino acid sequences from two or more proteins. The sequences from two or more proteins may be full or partial (i.e., fragments) of the proteins. Such fusion proteins may also have linking regions of amino acids between the portions corresponding to those of the proteins. Such fusion proteins may be prepared by recombinant methods, wherein the corresponding nucleic acids are joined through treatment with nucleases and ligases and incorporated into an expression vector. Preparation of fusion proteins is generally understood by those having ordinary skill in the art.

The phrase "polypeptide capable of binding to glutathione" refers to proteins, protein fragments, and synthetic polypeptides capable of binding to glutathione. Examples include glutathione-s-transferase and fragments thereof. Suitable fragments may be generated by gene amplification using 5' and 3' primers before translation or by proteolytic cleavage (see Table 1) after translation.

The term "coding region" refers to an open reading frame; i.e., a portion of a nucleic acid that has a sequence that would be translated to form a sequence of amino acids. The term "coding region" includes sequences of naturally occurring proteins as well as sequences resulting from modifications (insertions, deletions, mutations, disruptions) obtained through recombinant methods.

The term "linking region" refers to a sequence of amino acids between coding regions from different sources in a fusion protein. Typically, linking regions encode sites recognized by proteases and thus allow the expression products of the coding regions to be separated from each other.

The phrase "operatively linked to a promoter" means that the promoter is capable of directing the expression of the associated coding region. Coding regions for the fusion protein may also be operatively linked to other regulatory elements, such as enhancers.

The preferred embodiment employs a Gst sequence within commercially available expression vector pGEX-2T. This sequence is derived from Schistosoma japonicum. A number of species are known to produce active isoforms of Gst, all of which are useful in the present invention.

Coding regions for the fusion protein may be spliced into an expression vector by means well understood by those having ordinary skill in the art. Suitable expression vectors may be constructed using standard recombinant DNA techniques known in the art, many of which are described in Sambrook, et al., Molecular Cloning; A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Habor, NY (1989).

Suitable expression vectors in accordance with the present invention comprise a coding region for a polypeptide capable of binding to glutathione, along with an in-frame sequence for the protein to be isolated. The coding region for the protein to be isolated may be located upstream or downstream of the coding region for the glutathione-binding polypeptide. Preferred are expression vectors comprising one or more regulatory DNA sequenc s operativ ly linked to the DNA sequence coding for all or part of Gst.

Expression vectors useful in th pr sent invention typically contain an origin of replication, a promoter located 5' to (i. ., upstream of) th Gst fusion protein sequenc , which is followed by downstream transcription t rmination sequences, and th remaining vector. Control regions derived from a number of sources may b mployed in accordanc with th present invention. Suitable origins of replication include,

for example, the Col E1, the SV4O viral and the M13 orgins of replication. Suitable promoters include, for example, the cytomegalovirus promoter, the lac Z promoter, the gal 10 promoter and the Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) polyhedral promoter. Suitable termination sequences include, for example, SV40, lac Z and AcMNPV polyhedral polyadenylation signals. An expression vector as contemplated by the present invention is at least capable of directing the replication, and preferably the expression, of the nucleic acids encoding the fusion proteins.

The expression vectors may also include other DNA sequences known in the art; for example, stability leader sequences which provide for stability of the expression product; secretory leader sequences, which provide for secretion of the expression product; sequences that allow expression of the structural gene to be modulated (e.g., by the presence or absence of nutrients or other inducers in the growth medium); marking sequences, which are capable of providing phenotypic selection in transformed host cells (e.g., genes for neomycin, ampicillin, and hygromycin resistance and the like); and sequences that provide sites for cleavage by restriction endonucleases. All of these materials are known in the art and are commercially available.

The characteristics of the actual expression vector used must be compatible with the host cell to be employed. The vector thus may include sequences which allow expression in various types of host cells, including but not limited to prokaryotes, yeasts, fungi, plants and higher eukaryotes. For example, when expressing DNA sequences in a mammalian cell system, the expression vector should contain promoters isolated from the genome of mammalian cells, (e.g., mouse metallothionien promoter), or from viruses that grow in these cells (e.g., baculovirus promoter, vaccinia virus 7.5 K promoter).

Suitable commercially available expression vectors into which DNA sequences for the fusion proteins may be inserted include the mammalian expression vectors pcDNAI or pcDNA/Neo, the baculovirus expression vectors pBlueBac and pVL1393 (which is preferred), the prokaryotic expression vector pcDNAII and the yeast expression vector pYes2, all of which may be obtained from Invitrogen Corp., San Diego, CA. Preferred are commercially available vectors that already have Gst sequences included, such as pGEX-2T.

The present invention additionally concerns host cells containing an expression vector that comprises a DNA sequence coding for a Gst fusion protein. The host cells preferably contain an expression vector which comprises all or part of the DNA sequence for the protein to be isolated together with a DNA sequence for a polypeptide capable of binding glutathione. See, for example, the expression vector appearing in the Experimental Procedures hereinbelow, which is preferred. Further preferred are host cells containing an expression vector comprising one or more regulatory DNA sequences capable of directing the replication and/or the expression of and operatively linked to a DNA sequence coding for all or part of the fusion protein. Suitable host cells include both prokaryotic and eukaryotic cells. Suitable prokaryotic host cells include, for example, E. coli strains HB101, DH5α, XL1 Blue, Y1090 and JM101. Suitable eukaryotic host cells include, for example, Spodoptera frugiperda insect cells (which are preferred), COS-7 cells, human skin fibroblasts, and Saccharomyces cerevisiae cells.

Expression vectors may be introduced into host cells by various methods known in the art. For example, transfection of host cells with expression vectors can be carried out by the calcium phosphate precipitation method. However, other methods for introducing expression vectors into host cells, for example, electroporation, liposomal fusion, nuclear injection, and viral or phage infection can also be employed.

Once an expression vector has been introduced into an appropriate host cell, the host cell may be cultured under conditions permitting expression of large amounts of the fusion protein.

Figure 1: Construction of pBMS-I

A. Outline of the cloning procedure. The glutathione-s-transferase gene was cloned into the Bam H-1 site of the Sf9 expression vector pVL1393 to make the Gst fusion expression vector pBMS-1. The restriction map of the pBMS-1 polylinker, and the thrombin cleavage site are shown.

B. Schematic of the GstLck fusion junction. lck was joined to the Gst coding sequence using a Stu-1 site located 24 base pairs upstream of the lck intiation methionine codon.

Figure 2: Analysis of GstLck purified from Sf9 cells.

A. SDS-PAGE analysis and Coomassie staining pattern. Lane 1 shows the result from 50 µg of total protein from infected Sf9 cells; lane 2, 1 µg of purified GstLck; lane 3, 0.5 µg of thrombin-cleaved GstLck (recombinant $p56^{lck}$).

B. SDS-PAGE analysis of autophosphorylated GstLck. Lane 1 shows the result from autophosphorylation of GstLck; lane 2, autophosphorylation of recombinant $p56^{lck}$.

C. Western blot analysis of th sample used in panel B using a polyclonal rabbit anti-lck antibody. Lane 1 shows th result from GstLck; Lane 2, recombinant $p56^{lck}$.

Figure 3: Autophosphorylation of GstLck.

A. Western blot analysis of $p56^{lck}$. Lan 1 shows the r sult from immunoprecipitated $p56^{lck}$ from CEM-6 cells; lanes 2-4, GstLck from infected Sf9 cell lysat s purified using the following methods. Lane 2, immunoprecipitation using anti-lck polyclonal antibodies; lane 3, immunoprecipitation using anti-Gst polyclonal antibodies; lane 4, affinity purification using glutathione resin.

B. Analysis of the enzymatic activity of $p56^{lck}$or GstLck purified as outlined in panel A. Activity was assessed by autophosphorylation. The same protein samples and quantities were loaded as in panel A.

Figure 4: Phosphorylation of enolase by GstLck.

A. Phosphorylation of enolase as a function of GstLck concentration. Each reaction was carried out for 1 minute at 30 °C, with 3 µg of enolase as substrate, and varying amounts of GstLck. Lane 1 shows the result from 0 µg GstLck; Lane 2, 0.04 µg GstLck, lane 3, 0.08 µg GstLck; lane 4, 0.12 µg GstLck; lane 5, 0.2 µg GstLck; lane 6, 0.28 µg GstLck; lane 7, 0.36 µg GstLck; lane 8, 0.44 µg GstLck; lane 9, 0.52 µg GstLck.

B. Time course of enolase phosphorylation by GstLck. Each reaction was carried out at 30 °C, with 0.4 µg of GstLck, and 3 µg of enolase as substrate. Lane 1 shows the result after 0 minutes; lane 2, 0.5 minute; lane 3, 1 minute; lane 4, 2 minutes; lane 5, 3 minutes.

Figure 5: Phosphorylation of enolase by thrombin-cleaved GstLck.

A. Phosphorylation of enolase as a function of recombinant $p56^{lck}$concentration. Each reaction was carried out for 1 minute at 30 °C, with 3 µg of enolase as substrate, and varying amounts of recombinant $p56^{lck}$. Lane 1 shows the result from 0 µg $p56^{lck}$; lane 2, 0.01 µg $p56^{lck}$; lane 3, 0.02 µg $p56^{lck}$; lane 4, 0.03 µg $p56^{lck}$;lane 5, 0.05 µg $p56^{lck}$; lane 6, 0.07 µg $p56^{lck}$; lane 7, 0.09 µg $p56^{lck}$; lane 8, 0.11 µg $p56^{lck}$.

B. Time course of enolase phosphorylation by recombinant $p56^{lck}$. Each reaction was carried out at 30 °C, with 0.01 µg of recombinant $p56^{lck}$, and 3 µg of enolase as substrate. Lane 1 shows the result after 0 minutes; lane 2, 0.5 minutes; lane 3, 1 minute; lane 4, 2 minutes; lane 5, 3 minutes.

Experimental Procedures

Construction of $p56^{lck}$ expression vectors. A Stu-1 fragment from the mouse lck cDNA (Marth, J.D., Peet, R., Krebs, E.G., and Perlmutter, R. (1985) Cell 43, 393-404) was cloned into the filled-in Eco-R1 site of the vector pGEX-2T (Pharmacia). The resulting plasmid pGEX-lck is capable of expressing a glutathione-s-transferase/Lck (GstLck) fusion protein when transfected into E. coli cells. The GstLck coding sequence from pGEX-lck was amplified by PCR. The 5' PCR primer

5' TAT AAA TAT GTC CCC TAT ACT A 3'
(SEQ. ID. NO.: 3),

was synthesized on an Applied Biosystems, Inc. model 380A synthesizer. This primer hybridizes to the 5' region of the Gst coding sequence and encodes the ribosome binding site for the baculovirus polyhedrin gene. The 3' PCR primer,

5' CGT CAG TCA GTC ACG AT 3'
(SEQ. ID. NO.: 4),

hybridizes to sequences immediately 3' to the polylinker of pGEX-2T. This primer pair can be used to amplify any sequence cloned into the polylinker of pGEX-2T as a Gst/insert fusion. The amplified GstLck coding sequence was cloned into the vector pCR1000 (InVitrogen, Inc.) resulting in the plasmid pCR1000-GstLck. The pCR1000 vector was designed for easy cloning of PCR-amplified DNA, and was used as an intermediate cloning vector. A Not-1, Bgl-II fragment from pCR1000-GstLck containing GstLck coding sequence was cloned into the Not-I, Bgl-II sites of pVL1393. Lukow, V.A., and Summers, M.D. (1988) Virology 167, 56-71. Th resulting plasmid, pVL1393-GstLck (A.T.C.C. Accession No. ___, American Type Culture Collection, 12301 Parklawn Driv , Rockville, Maryland 20852-1776) was used to produce a recombinant baculovirus in Spodoptera frugiperda 9 (Sf9) cells following standard procedures. Summers, M.D., and Smith, G.E. (1987). A Manual for baculovirus vectors and insect cell culture procedur s, T xas

A&M bulletin No. 1555, (College Station, Texas Agricultural Experimental Station and T xas A&M University), 10-39. The cloning scheme used for the construction of pBMS-I is outlined in figure 1A. The PCR primers used are the same described above.

Purification of GstLck from Sf9 cells. A 500 mL spinner culture of infected Sf9 cells in Excell-400 medium (JRH Biosciences) was harvested 48 hours after infection by centrifugation at 4°C for 5 minutes. The cells were lysed in 50 mL of cold 50 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 1%-(vol/vol) NP-40, 1 mM PMSF, 0.1 mg/mL aprotinin, 0.1 mg/mL leupeptin, 1 mM NaF, and 1 mM Na_3VO_4 -(lysis buffer). Insoluble material was removed by centrifugation at 10,000 x g for 10 minutes at 4°C. The resulting cell lysate was determined to have a protein concentration of 9.5 mg/mL using the Coomassie Protein Assay Reagent (Pierce).

The GstLck protein was purified by a one-step affinity chromatograpy procedure using glutathione resin as described by the manufacturer (Pharmacia). For this experiment, 50 mg of Sf9 cellular lysate containing the GstLck protein was added to a 2-mL glutathione column and the unbound material removed by washing with 50 mL of lysis buffer. Bound proteins were eluted from the column with 2 column volumes of lysis buffer containing 5 mM glutathione. Eluted protein was diluted to 15 mL with lysis buffer and concentrated using a Centriprep 30 Concentrator unit (Amicon, Inc.). Two additional dilutions and concentrations were performed to remove the remaining glutathione. The concentrated protein was adjusted to 10% glycerol and stored at -70°C. This procedure yielded 28.0 mg of greater than 99% pure GstLck as determined by SDS-PAGE and Coomassie Blue staining analysis.

To obtain $p56^{lck}$ protein lacking the Gst peptide sequences, GstLck was digested with the proteolytic enzyme thrombin to generate cleaved $p56^{lck}$ ($cp56^{lck}$). For this procedure 5 mg of thrombin was added to 20 mg of purified GstLck in a volume of 50 mL lysis buffer, containing 2.5 mM $CaCl_2$ for 1 hour at 25°C. To remove uncleaved GstLck and cleaved Gst, the products were mixed with 20 mL of glutathione resin. The glutathione resin was removed by centrifugation leaving the $cp56^{lck}$ in the supernatant. The yield from this procedure was approximately 5 mg of recombinant $p56^{lck}$ which was stored in 10% glycerol at -70°C.

Immune-complex protein kinase assays. Analysis of protein kinase activity conducted on immune-complexes was carried out as previously described. Veillette, A., Horak, I.D., Horak, E.M., Bookman, M.A., and Bolen, J.A. (1988) Mol. Cell. Biol. 8, 4353-4361. Briefly, immune-complexes formed from cellular lysates and the indicated antisera were collected by the addition of formalin-fixed Staphyloccocus aureus -(Pansorbin, Calbiochem) and washed extensively in lysis buffer. Protein kinase reactions were initiated by the addition of 30 mL kinase buffer (20 mM MOPS pH 7,5 mM $MnCl_2$, 1 mM ATP) containing 12.5 µCi [γ-^{32}P]-ATP (3000Ci/mmol, New England Nuclear). The reactions were allowed to proceed for 5 minutes at room temperature and stopped by addition of an equal volume of 2X SDS loading buffer (0.125 M Tris-HCl pH 6.8, 4% (weight/vol) SDS, 20% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol). The phosphorylated products in SDS loading buffer were heated for 5 minutes at 90°C and analyzed by SDS-PAGE and autoradiography. The ^{32}P-labeled bands of interest were excised from the gel and counted in a Beckman LS6000TA liquid scintillation counter.

Soluble protein kinase assays. The enzymatic activity of GstLck and $cp56^{lck}$ were evaluated by their capacity to phosphorylate the Lck exogenous substrate rabbit muscle enolase (Sigma). To determine the time course of enolase phosphorylation, 3 µg of GstLck or 1 µg of $cp56^{lck}$ was added to 100 µl of kinase buffer containing 12 µg enolase and 25 µCi [γ-^{32}P] ATP and the reactions were conducted at 30°C for the indicated times. At each point, 10 µL of the reaction mix was removed, added to 30 µL of 2X SDS loading buffer and heated for 5 minutes at 90°C. The reaction products were analyzed by SDS-PAGE and autoradiography. The bands corresponding to enolase were excised from the gel and counted by liquid scintillation spectroscopy. To determine the K_m for enolase, serial dilutions of enolase were added to kinase buffer containing 5 µCi [γ-^{32}P]-ATP, and either 0.1 µg of GstLck or 0.01 µg of $cp56^{lck}$ were added per reaction. Reaction conditions and the counts incorporated into enolase were determined as described above. For the K_m determination of ATP, a 1:10 dilution of [γ-^{32}P]-ATP was added to kinase buffer containing 3 µg enolase. For each ATP dilution, 1 µg of $cp56^{lck}$ was added in a total volume of 30 µL and reacted for 30 seconds at 30°C. Reactions were stopped by addition of 30 µL of 2X SDS loading buffer and heated to 90°C. The reaction products were analyzed by SDS-PAGE, the phosphorylated proteins visualized by autoradiography, and ^{32}P incorporation determined by liquid scintillation spectroscopy of the excised bands.

Other biochemical assays and materials. Lck immunoblot analysis was conducted as previously d scribed using rabbit anti-Lck antis ra. Veillette, A., Bookman, M.A., Horak, E.M., and Bolen, J.A. (1988) Cell 55, 301-308. Partial proteolytic peptid analysis using Staphylococcus aureus V8 protease (Pierce) has also been pr viously described. V illett , A., Horak, I.D., Horak, E.M., Bookman, M.A., and Bolen, J.A. (1988) Mol. Cell. Biol. 8, 4353-4361 ; Marth, J.D., Cooper, J.A., King, C.S., Ziegler, S.F., Tinker, D.A, Overell,

R.A, Krebs, E.G., and Perlmutter, R.M. (1988) Mol. Cell. Biol. 8, 540-550. Th human T-cell lymphoma cell line CEM was grown in RPMI 1640 m dia supplem nted with 10% (vol/vol) fetal bovine serum and antibiotics (penicillin/streptomycin). For immunoprecipitation experiments, the cells were washed in phosphate buffered saline, collected by centrifugation, lysed in lysis buffer, and adjusted to 1 mg/ml prior to addition of anti-Lck antisera. Antisera directed against Gst was prepared by immunization of rabbits with purified Gst. Antisera directed against Lck amino acids 39-58 has been previously described. Veillette, A., Bookman, M.A., Horak, E.M., and Bolen, J.A. (1988) Cell 55, 301-308.

Results

Construction of expression vectors. Figure 1A outlines the cloning strategy used to create the expression vector pBMS-I. The Gst coding sequence from pGEX-2T was cloned by PCR amplification, and ligated into the baculovirus expression vector pVL1393. The 5' PCR primer was designed to optimize translation of the Gst coding sequence in Sf9 cells. This was accomplished by changing the sequence surrounding the initiation methionine of Gst to encode the ribosomal binding site of the baculovirus polyhedrin gene. The pBMS-I polylinker contains 9 unique cloning sites, and can be used to make a recombinant baculovirus that expresses inserts as a Gst fusion protein in Sf9 cells.

The fusion junction of the GstLck coding sequences cloned into pVL1393 is schematically shown in figure 1B. The thrombin cleavage site is also indicated. This plasmid pVL1393-GstLck was used to make a recombinant baculovirus that expressed high levels of the GstLck fusion protein in Sf9 cells. Thrombin cleavage of GstLck protein resulted in a recombinant $p56^{lck}$ ($cp56^{lck}$) molecule containing an additional 13 amino acids at the Lck amino-terminus. These additional amino acids had no apparent affect on the in vitro enzymatic activity of recombinant $p56^{lck}$. This was determined by comparing the immune-complex protein kinase activities of $cp56^{lck}$ with that of wild-type $p56^{lck}$ expressed in Sf9 cells.

Purification of GstLck from Sf9 cells. Total detergent lysates were made from Sf9 cells expressing the GstLck fusion protein as outlined in Experimental Procedures. Lysate containing GstLck was bound to a glutathione-sepharose column and eluted with 5 mM glutathione in lysis buffer. The glutathione-bound products from this column were analyzed by Coomassie staining following fractionation on SDS polyacrylamide gels. As shown in figure 2A, a single polypetide of approximately 83 kDa was observed which corresponds to the expected size for the GstLck fusion protein. Following thrombin cleavage (figure 2A, lane 3), the recombinant Lck protein was observed to migrate as two closely spaced bands at approximately 56 kDa.

Functional analysis of GstLck and $cp56^{lck}$. To evaluate the kinase activity of the purified GstLck and $cp56^{lck}$ proteins, protein kinase assays were performed. The results of these reactions (figure 2B) demonstrated that purified GstLck and $cp56^{lck}$ maintained their autophosphorylation capacity. As expected, no kinase activity was detected in purified preparations of Gst. The data shown in figure 2C represents the corresponding Lck immunoblot using polyclonal rabbit antibodies against the $p56^{lck}$ unique region. Based on the relative amounts of Lck protein detected in the kinase reactions, it appears that the specific activity of the $cp56^{lck}$ may be slightly higher than that of the GstLck fusion protein. Anti-phosphotyrosine immunoblot analysis of similar reaction products generated using non-radioactive ATP demonstrated that the autophosphorylation products (as well as the phosphorylation of exogenous protein substrate enolase used in other experiments) were phosphorylated on tyrosine residues. Additionally, partial V8 peptide analysis of the autophosphorylation products of the GstLck and $cp56^{lck}$ reactions yielded major V8 phosphopeptides indistinguishable from that of T-cell derived $p56^{lck}$ autophosphorylated in immune-complex kinase assays.

The level of GstLck enzymatic activity was also compared to that of wild type $p56^{lck}$ immunoprecipitated from T-cell detergent lysates. For these experiments, GstLck was precipitated from infected Sf9 detergent lysates with anti-Lck antisera, anti-Gst antisera, or with glutathione-Sepharose beads. The $p56^{lck}$ from T-cell lysates was immunoprecipitated with anti-Lck antisera. The various complexes were washed extensively with lysis buffer and divided into two equal aliquots. One aliquot was used to perform protein kinase assays (figure 3B) while the other aliquot was used for Lck immunoblot analysis (figure 3A). The results of this experiment demonstrate that precipitation of the GstLck protein using either antibodies or glutathione beads yielded molecules with similar specific activities as assessed by autophosphorylation. Comparison with $p56^{lck}$ derived from T-cells showed that th specific activity of the Sf9 derived GstLck protein was significantly higher.

To furth r charact rize the kinetic parameters of GstLck and $cp56^{lck}$, kinase activity of the fusion protein and cleaved enzym was studied using rabbit muscle enolase as an xogenous substrate. As shown by the data presented in figur 4, th phosphorylation of enolase by GstLck was found to be both tim and

concentration dependent. Similar results w re obtained for cp56^{lck} (figure 5). The K_m and V_{max} values for ATP and enolase w re det rmined using a reaction time of 30 seconds and the results summarized in Table I. The affinity of cp56^{lck} for enolase was found to be approximately 10-fold higher then that of GstLck. More critically the K_m and V_{max} values determined for cp56^{lck} are comparable to values obtained for other src family members.

Attempts to produce functional GstLck in E. coli were unsuccessful. The resulting fusion protein was expressed, but it lacked detectable protein kinase activity and was found to be insoluble in detergents. The latter feature is common to expression of many eukaroytic proteins in bacteria. Marston, A.O. (1986) J. Biochem. **240**, 1-12; Miller, D.W., Saher, P., and Miller, L.K. (1986) in Genetic Engineering, vol. 8, pp. 277-298, Plenum, New York; Miller, L.K. (1989) in Ann. Rev. Microbiol. **42**, 177-199. Among the advantages of expression of eukaryotic proteins in Sf9 cells is the capacity of these cells to allow protein folding and post-translational modification that maintain protein solubility. In the case of Lck, expression of the wild-type p56^{lck} in Sf9 cells using conventional baculovirus expression vectors has shown that Lck is myristylated and phosphorylated on serine and threonine residues. Thomas, J.E., Soriano, P., and Brugge, J.S.. (1991) Science **254**,.568-571. Since Lck in this system is expressed as a fusion protein with Gst at the aminoterminus, it is unlikely that myristylation occurs. We have not determined whether the GstLck is phosphorylated on serine or threonine residues.

Discussion

The lck coding sequences were ligated downstream from the Gst coding region in-frame to yield a plasmid capable of encoding a Gst-p56^{lck} fusion protein. The p56^{lck} produced in this manner was found to be a highly active protein kinase, and exhibited the expected biochemical properties of a member of the src family.

Analysis of both the GstLck fusion protein as well as the cp56^{lck} indicated that each retained significant protein tyrosine kinase activity as measured by autophosphorylation and tyrosine phosphorylation of the exogenous substrate rabbit muscle enolase. Importantly, the Gst sequences, whether fused to Lck or following cleavage from the kinase with thrombin, were not phosphorylated in immune-complex kinase assays or in kinase assays conducted in solution. Both the GstLck and the cp56^{lck} were found to have substantially higher specific activities than p56^{lck} derived from T-cells when measured by immune-complex protein kinase assays. The altered specific activity is likely to be the result of diminished carboxy-terminal tyrosine (tyrosine 505) phosphorylation for Lck in Sf9 cells although we have not determined the phosphorylation sites of Lck in these cells. Veillette, A., Horak, I.D., Horak, E.M., Bookman, M.A., and Bolen, J.A. (1988) Mol. Cell. Biol. **8**, 4353-4361; Marth, J.D., Cooper, J.A., King, C.S., Ziegler, S.F., Tinker, D.A., Overell, R.A., Krebs, E.G., and Perlmutter, R.M. (1988) Mol. Cell. Biol. **8**, 540-550. The lack of tyrosine 505 phosphorylation of Lck, like that observed with Sf9-derived pp60^{c-src} (Morgan, D.O., Kaplan, J.M., Bishop, J.M., and Varmus, H.E. (1989)Cell **57**, 775-786), is probably attributable to the absence of expression of other tyrosine protein kinases such as Csk that are thought to phosphorylate the Src class of kinases at this site. Okada, M., and Nakagawa, H. (1989) J. Biol. Chem. **264**, 20886-20893; Okada, M., and Nakagawa, H. (1988) Biochem. Biophys. Res. Commun. **154**, 796-802.

From 50 mg of total Sf9 protein lysate, the foregoing procedure purified 280 mg of greater than 99% pure (by silver and Coomassie staining) recombinant p56^{lck}. From one liter of infected Sf9 cells, this system produced approximately 8-10 mg of purified recombinant Lck.

The foregoing procedures were also used to produce GstLynB, GstSyk, GstBlk, GstFyn, and GstYes fusion proteins with comparable results and yields to that reported here for Lck.

The abbreviations used throughout this specification are defined as follows.

ATP	adenosine triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
MOPS	(3-[N-morpholino]propanesulfonic acid)
PCR	polymerase chain reaction
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate

The gene for GST can be cleaved by enzymes at the positions shown in Table 1. Such nucleic acid fragments can be used to generate partial Gst polypeptides in the fusion proteins of the present invention.

Table I

11	EcoN1	208	Msc1	495	Asu1	667	Aci1
13	Bfa1	208	Pal1	495	Ava11	668	Alw1
13	BsiY1	216	Mae11	495	Bme181	669	Acc11
13	Bsl1	226	Alu1	495	BsiZ1	669	Bsh1236 1
13	Mae1	239	Af1111	495	Cfr13I	669	Bsp501
13	Rma1	243	N1a111	495	Eco47I	669	BstU1
17	BsmF 1	243	Nsp75241	495	Eco471	669	FnuD11
26	EcoR1*	243	NspB1	495	N1a111	669	Mvn1
26	Tsp509 1	243	Nsp1	495	NspB11	669	Tha1
29	Mse1	287	Bsq1	495	Nsp1V	673	BamB1
33	Asu1	292	BsrB 1	495	Sau96I	673	BspA1
33	BsiZ1	319	Taq1	495	Sin1	673	BstY1
33	Cfr13I	319	TthHB81	497	BscB1	673	Dpn11
33	Dra11	323	EcoR1*	497	N1aIV	673	Kzo91
33	Eco01091	323	Tsp509 1	501	SfaN1	673	Mbo1
33	Nsp1V	333	BsmA1	506	DsaV	673	Mf11
33	Sau96I	367	Dde1	506	EcoR11	673	Nde11
35	BsuR1	375	Alu1	508	Apy1	673	Sau3A1
35	Hae111	394	Asp7001	508	BsiL1	673	Xho11
35	Pal1	394	Xmn1	508	BstN1	675	BscB1
36	Pss1	398	Asu11	508	BstO1	675	Dpn1
51	Taq1	398	Bpu141	508	Mva1	675	N1aIV
51	TthHB81	398	BsiC1	508	ScrF1	677	BsaJ1
65	Bcq1	398	Bsp1191	523	EcoR1*	677	Bsa11
80	Eam11041	398	BstB1	523	Fok1	677	DsaV
80	Ear1	398	Csp451	523	Tsp509 1	677	Sec1
80	Ksp6321	398	Lsp1	536	Mse1	678	Aqu1
85	Mbo11	398	Nsp7524V	537	Aha111	678	Ava1
95	Msl 1	398	NspV	537	Dra1	678	Bco1
97	Mbo11	398	Sfu1	543	Mae11	678	BsaJ1
102	Hin61	398	Taq1	553	Alu1	678	Bsa11
102	HinP11	398	TthHB81	563	EcoR1*	678	Cfr91
102	HinP1	402	BspA1	563	Tsp509 1	678	DsaV
104	Acc11	402	Dpn11	573	Csp61	678	Eco881
104	Bsh1236 1	402	Kzo91	574	Afa1	678	PspA1
104	Bsp501	402	Mbo1	574	Rsa1	678	Sec1
104	BstU1	402	Nde11	574	Sca1	678	Xcy1
104	Cfo1	402	Sau3A1	602	N1a111	678	Xma1
104	FnuD11	404	Dpn1	603	BsuR1	679	Aha1
104	Hha1	412	Mbo11	603	Hae111	679	Bcn1
104	Mvn1	427	Mse1	603	Pal1	679	Bap11
104	Tha1	428	Aha111	610	BsiY1	679	Hpa11
121	AciI	428	Dra1	610	Bsl1	679	Msp1
124	Hph1	428	SwaI	615	BspM1	679	Nci1
139	EcoR1*	434	Fba1	615	Mwo 1	679	ScrF1
139	Tsp509 1	434	Fok1	625	Mae11	680	Aha1
154	Mbo11	435	Bc11	629	Fok1	680	Bcn1
188	Mse1	435	BsiQ1	636	AciI	680	Nci1
190	EcoR1*	435	BspA1	656	Mn11	680	ScrF1
190	Tsp509 1	435	Dpn11	657	BspA1	680	Sma1
193	Hph1	435	Kzo91	657	BstY1	681	Alw1
193	Mse1	435	Mbo1	657	Dpn11	683	Apo1
205	BsmA1	435	Nde11	657	Kzo91	683	EcoR1*
206	Cfr1	435	Sau3A1	657	Mbo1	683	EcoR1
206	Eae1	437	Dpn1	657	Mf11	683	Tsp509 1
208	Ba11	440	Fba1	657	Nde11		
208	BsuR1	441	Mae111	657	Sau3A1		
208	Hae111	442	N1a111	657	Xho11		
		445	Hph1	659	Dpn1		
		462	N1a111	665	Alw1		
		478	Hga1	665	BscB1		
		495	Af11	665	N1aIV		

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Spana, Carl
Fargnoli, Joseph
Bolen, Joseph B.

(ii) TITLE OF INVENTION: PROTEIN EXPRESSION SYSTEM

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Burton Rodney
(B) STREET: P.O. Box 4000
(C) CITY: Princeton
(D) STATE: New Jersey
(E) COUNTRY: U.S.A.
(F) ZIP: 08543-4000

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Gaul, Timothy J.
(B) REGISTRATION NUMBER: 33,111
(C) REFERENCE/DOCKET NUMBER: DC25

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (609) 252-5901
(B) TELEFAX: 609) 252-4526

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 693 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..693

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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 1               5                  10                  15

ACT CGA CTT CTT TTG GAA TAT CTT GAA GAA AAA TAT GAA GAG CAT TTG      96
Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu His Leu
            20                  25                  30

TAT GAG CGC GAT GAA GGT GAT AAA TGG CGA AAC AAA AAG TTT GAA TTG     144
Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu
        35                  40                  45

GGT TTG GAG TTT CCC AAT CTT CCT TAT TAT ATT GAT GGT GAT GTT AAA     192
Gly Leu Glu Phe Pro Asn Leu Pro Tyr Tyr Ile Asp Gly Asp Val Lys
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TTA ACA CAG TCT ATG GCC ATC ATA CGT TAT ATA GCT GAC AAG CAC AAC     240
Leu Thr Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala Asp Lys His Asn
65                  70                  75                  80

ATG TTG GGT GGT TGT CCA AAA GAG CGT GCA GAG ATT TCA ATG CTT GAA     288
Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu
                85                  90                  95

GGA GCG GTT TTG GAT ATT AGA TAC GGT GTT TCG AGA ATT GCA TAT AGT     336
Gly Ala Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser
            100                 105                 110

AAA GAC TTT GAA ACT CTC AAA GTT GAT TTT CTT AGC AAG CTA CCT GAA     384
Lys Asp Phe Glu Thr Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu
        115                 120                 125

ATG CTG AAA ATG TTC GAA GAT CGT TTA TGT CAT AAA ACA TAT TTA AAT     432
Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys Thr Tyr Leu Asn
    130                 135                 140
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GGT GAT CAT GTA ACC CAT CCT GAC TTC ATG TTG TAT GAC GCT CTT GAT      480
Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp
145                 150                 155                 160

GTT GTT TTA TAC ATG GAC CCA ATG TGC CTG GAT GCG TTC CCA AAA TTA      528
Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu
                165                 170                 175

GTT TGT TTT AAA AAA CGT ATT GAA GCT ATC CCA CAA ATT GAT AAG TAC      576
Val Cys Phe Lys Lys Arg Ile Glu Ala Ile Pro Gln Ile Asp Lys Tyr
            180                 185                 190

TTG AAA TCC AGC AAG TAT ATA GCA TGG CCT TTG CAG GGC TGG CAA GCC      624
Leu Lys Ser Ser Lys Tyr Ile Ala Trp Pro Leu Gln Gly Trp Gln Ala
        195                 200                 205

ACG TTT GGT GGT GGC GAC CAT CCT CCA AAA TCG GAT CTG GTT CCG CGT      672
Thr Phe Gly Gly Gly Asp His Pro Pro Lys Ser Asp Leu Val Pro Arg
    210                 215                 220

GGA TCC CCG GGA ATT CAT CGT                                         693
Gly Ser Pro Gly Ile His Arg
225                 230
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 231 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu
        35                  40                  45

Gly Leu Glu Phe Pro Asn Leu Pro Tyr Tyr Ile Asp Gly Asp Val Lys
    50                  55                  60

Leu Thr Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala Asp Lys His Asn
65                  70                  75                  80
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Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu
                    85                  90                  95

Gly Ala Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser
                100                 105                 110

Lys Asp Phe Glu Thr Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu
            115                 120                 125

Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys Thr Tyr Leu Asn
        130                 135                 140

Gly Asp His Val Thr His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp
145                 150                 155                 160

Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu
                165                 170                 175

Val Cys Phe Lys Lys Arg Ile Glu Ala Ile Pro Gln Ile Asp Lys Tyr
            180                 185                 190

Leu Lys Ser Ser Lys Tyr Ile Ala Trp Pro Leu Gln Gly Trp Gln Ala
            195                 200                 205

Thr Phe Gly Gly Gly Asp His Pro Pro Lys Ser Asp Leu Val Pro Arg
        210                 215                 220

Gly Ser Pro Gly Ile His Arg
225                 230
```

Claims

1. An expression vector, comprising:
   (a) a first coding region, which codes for a polypeptide capable of binding to gluthathione, operatively connected to a promoter,
   (b) a second coding region in-frame with the first coding region, and
   (c) at least one restriction site between the first and second coding regions;
   wherein a fusion protein of the first and second coding regions would result from expression of the vector.

2. A host cell, comprising the vector of Claim 1.

3. A process for isolating and purifying a protein, which comprises:
   (a) treating the host cell of Claim 2 under conditions allowing expression of the vector, whereby a fusion protein of the first and second coding regions will be expressed;
   (b) exposing proteins from the host cell to glutathione resin, whereby the fusion protein will adhere to the resin; and
   (c) cleaving the expression product of the second coding region from the resin-bound fusion protein.

4. A process for expressing a nucleic acid sequence, which comprises:
   (a) inserting the nucleic acid sequence into a baculovirus expression vector in-frame with a first coding region for a polypeptide capable of binding to glutathione wherein the first coding region is operatively linked to a promot r;
   (b) placing th vector into a host c ll;
   (c) treating the host cell under conditions allowing expression of the vector, resulting in expression of a fusion protein of th first coding region and th sequence inserted in step (a);

(d) exposing prot ins from the host cell to glutathione resin, wher by the fusion protein will adh re to the resin; and
(e) treating the adhered fusion protein with a protease to release the expression product of the nucleic acid sequence from the resin.

5. The expression vector of Claim 1, wherein the promoter is a baculovirus promoter.

6. The host cell of Claim 2, wherein the cell is a Spodoptera frugiperda cell.

7. The host cell of Claim 2, wherein the cell is a Spodoptera frugiperda cell and the expression vector comprises a baculovirus promoter.

8. The process of Claim 3, wherein the host cell is a Spodoptera frugiperda cell and the promoter is a baculovirus promoter.

9. The process of Claim 4, wherein the host cell is a Spodoptera frugiperda cell and the promoter is a baculovirus promoter.

10. The host cell of Claim 2, wherein the cell is an Sf9 cell.

11. The host cell of Claim 2, wherein the cell is an Sf9 cell and the promoter is a baculovirus promoter.

12. The process of Claim 3, wherein the host cell is an Sf9 cell and the promoter is a baculovirus promoter.

13. The process of Claim 4, wherein the host cell is an Sf9 cell and the promoter is a baculovirus promoter.

14. The vector of Claim 1, wherein the second coding region is Lck, LynB, Syk, Blk, Fyn, or Yes.

15. The host cell of Claim 2, wherein the second coding region is Lck, LynB, Syk, Blk, Fyn, or Yes..

16. The process of Claim 3, wherein the second coding region is Lck, LynB, Syk, Blk, Fyn, or Yes..

17. The process of Claim 4, wherein the target protein is Lck protein.

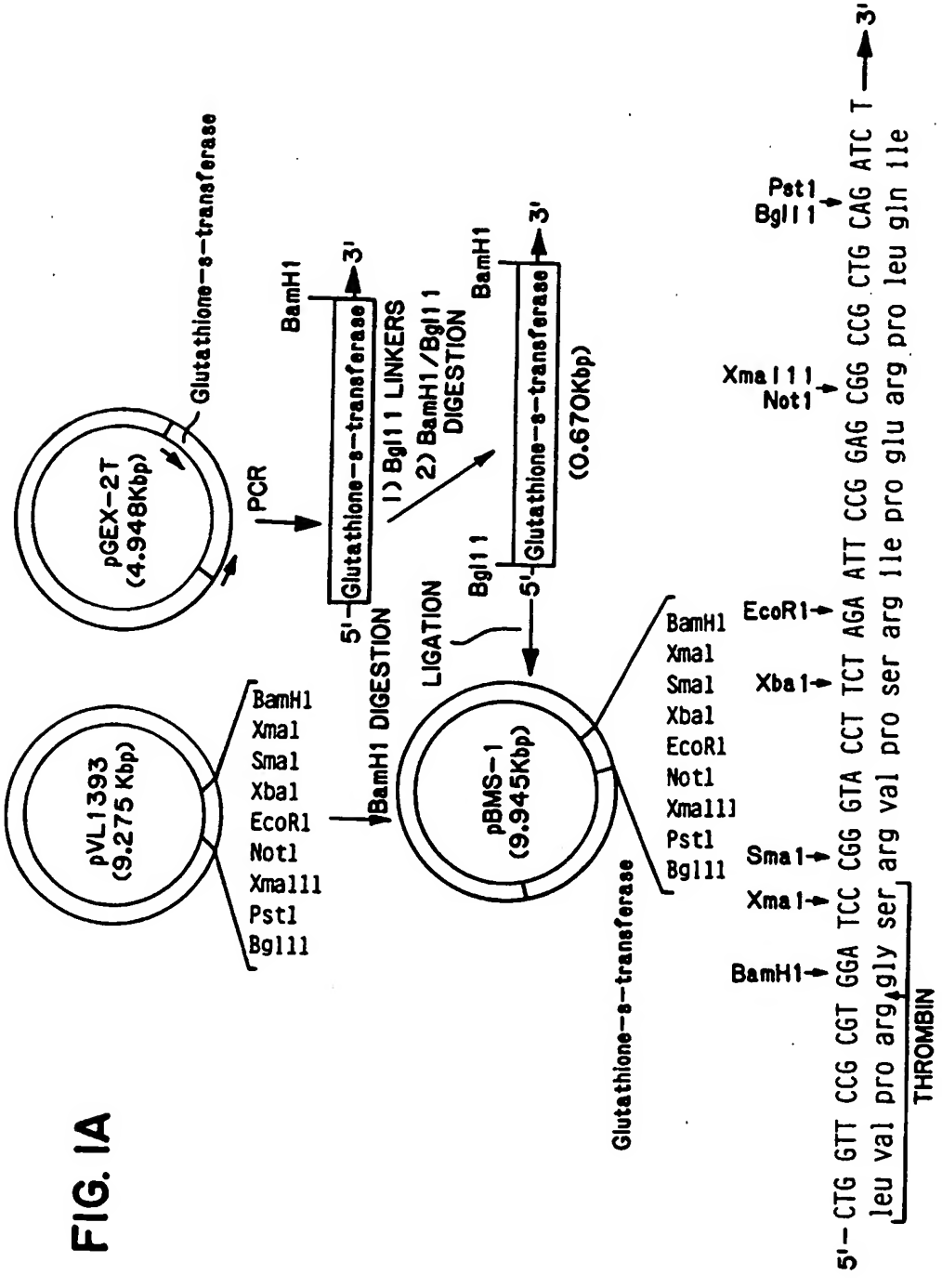
18. The expression vector of Claim 1, wherein the first coding region encodes glutathione-s-transferase.

19. The host cell of Claim 2, wherein the first coding region encodes glutathione-s-transferase.

20. The process of Claim 3, wherein the first coding region encodes glutathione-s-transferase.

21. The process of Claim 4, wherein the first coding region encodes glutathione-s-transferase.

FIG. IA


pGEX-2T
(4.948Kbp)
Glutathione-s-transferase
PCR
BamH1
3'
Glutathione-s-transferase
5'
1) Bgl11 LINKERS
2) BamH1/Bgl11
DIGESTION
BamH1
3'
Glutathione-s-transferase
(0.670Kbp)
5'
Bgl11
LIGATION
BamH1 DIGESTION
pVL1393
(9.275 Kbp)
BamH1
Xmal
Smal
Xbal
EcoR1
Not1
Xmalll
Pstl
Bglll
pBMS-1
(9.945Kbp)
Glutathione-s-transferase
BamH1
Xmal
Smal
Xbal
EcoR1
Not1
Xmalll
Pstl
Bglll
EcoR1
Xba1
Sma1
Xma1
BamH1
Pst1
Bgl11
Xma111
Not1
5'- CTG GTT CCG CGT GGA TCC CGG GTA CCT TCT AGA ATT CCG GAG CGG CCG CTG CAG ATC T -3'
leu val pro arg gly ser arg val pro ser arg ile pro glu arg pro leu gln ile
THROMBIN

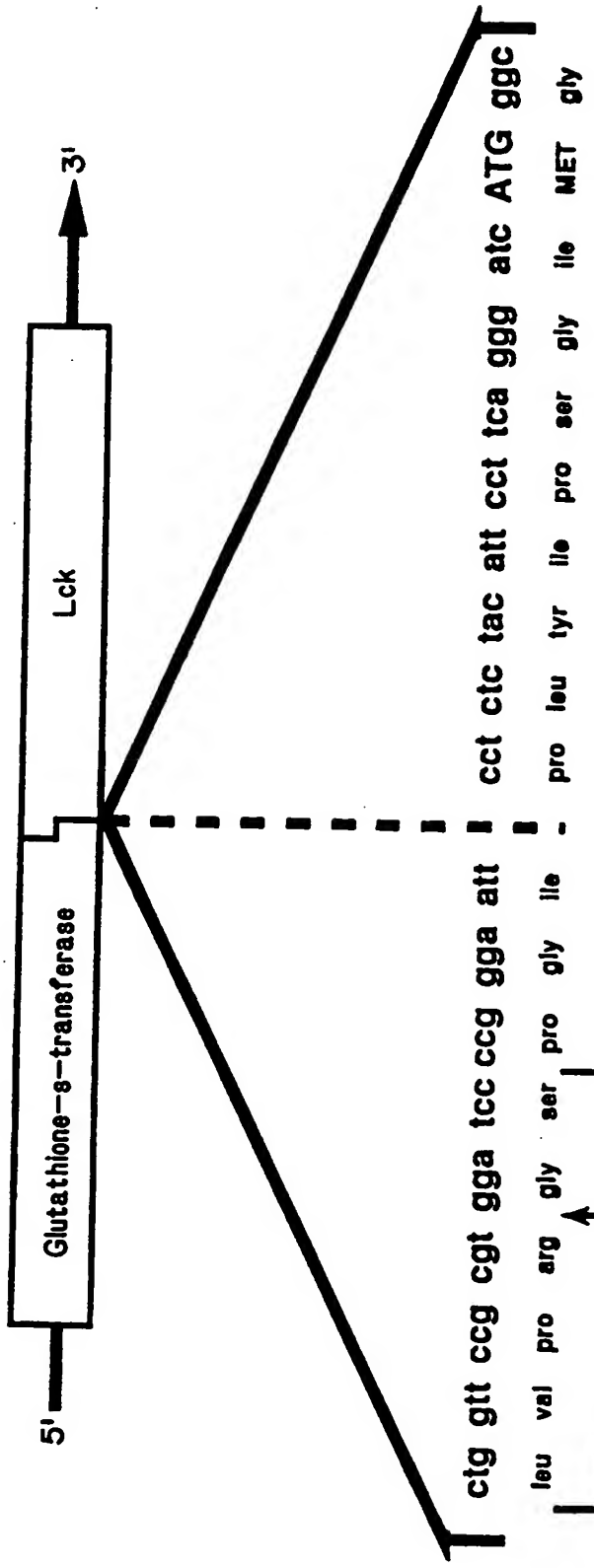

5'
Glutathione-s-transferase
Lck
3'
ctg gtt ccg cgt gga tcc ccg gga att
leu val pro arg gly ser pro gly ile
THROMBIN
cct ctc tac att cct tca ggg atc ATG ggc
pro leu tyr ile pro ser gly ile MET gly

FIG. 1B

FIG. 2A

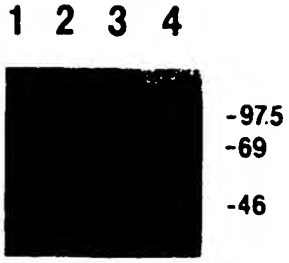
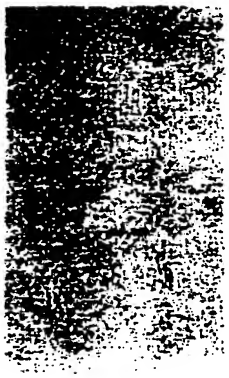


FIG. 2B

FIG. 2C



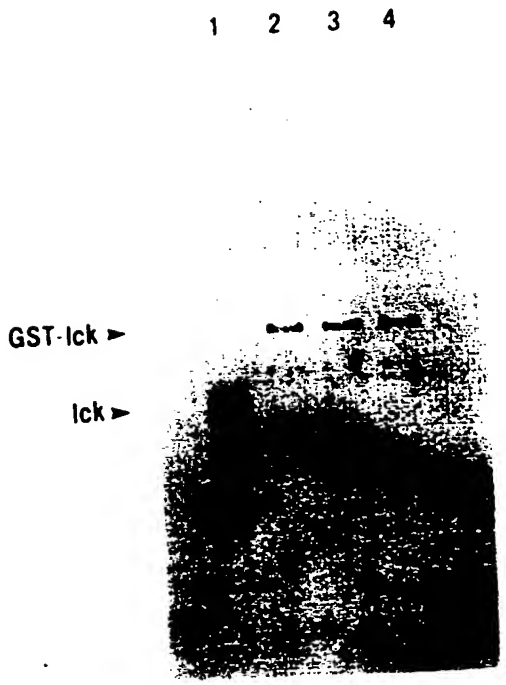


FIG. 3A

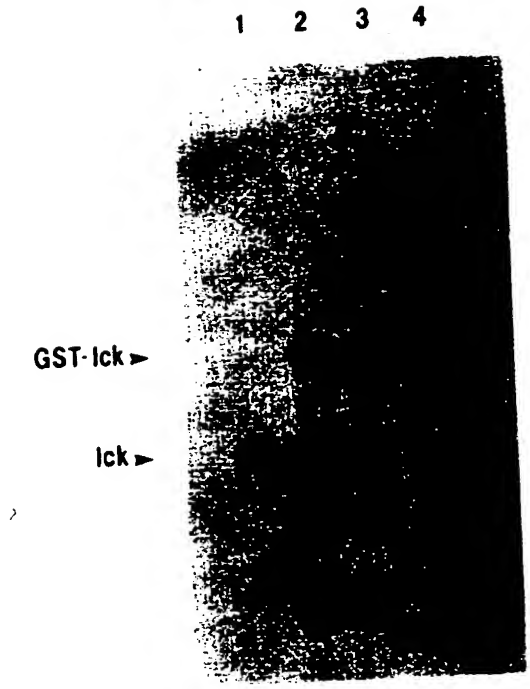


FIG. 3B

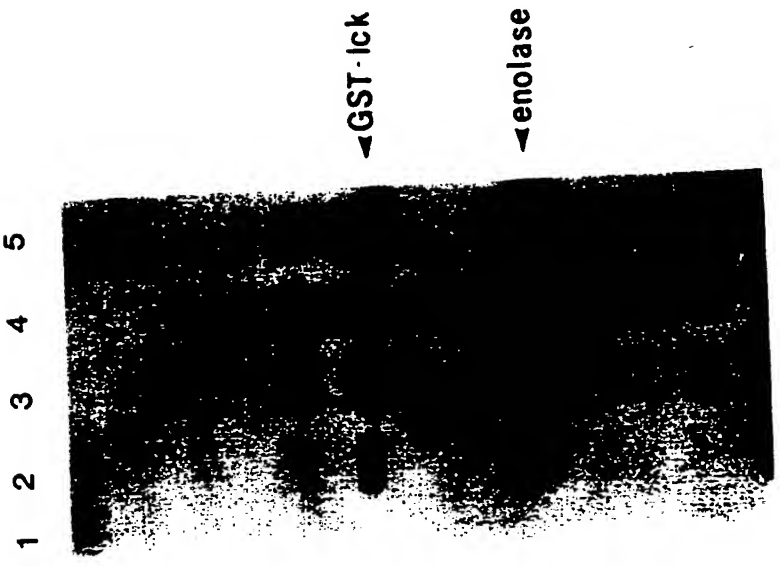


FIG. 4B

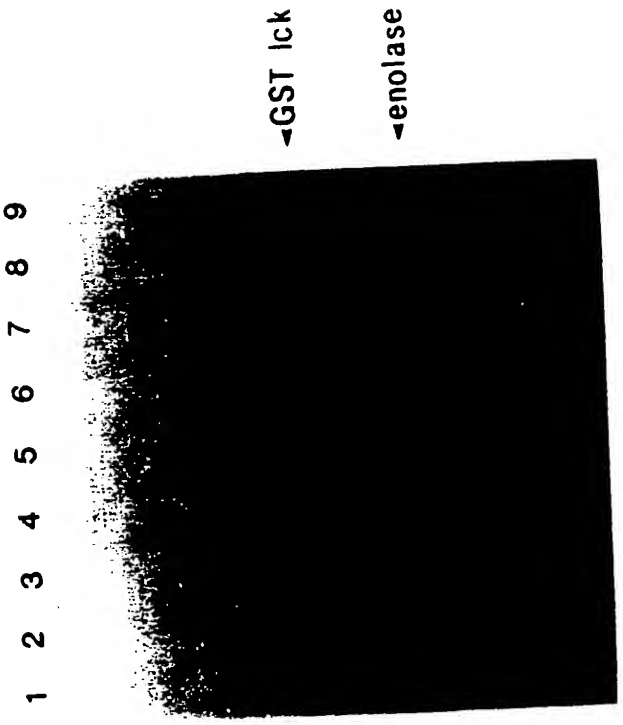


FIG. 4A

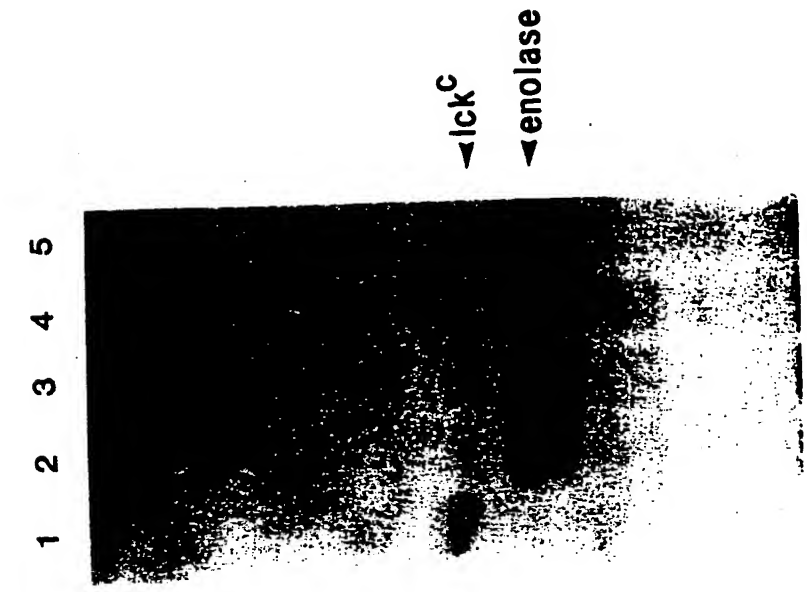


FIG. 5B

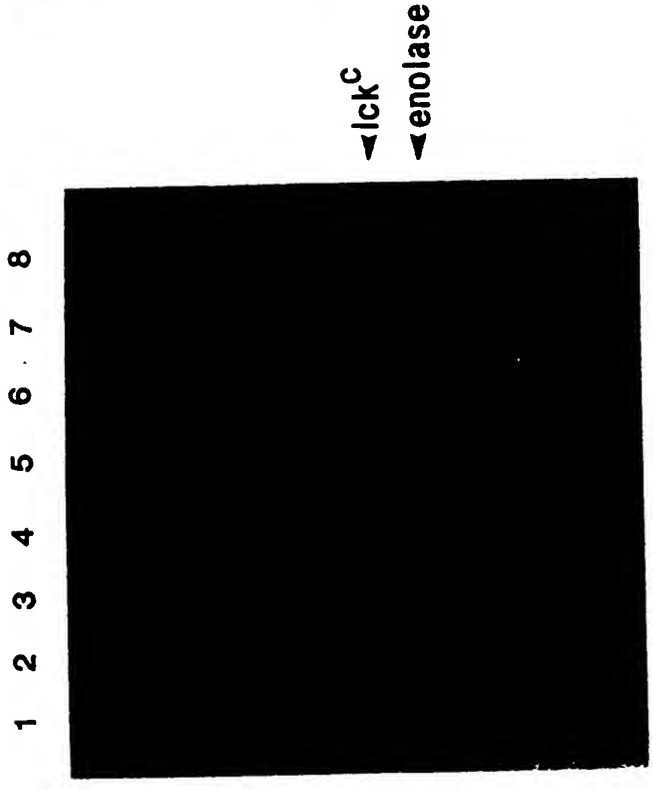


FIG. 5A